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Original Paper

Involvement of Ca^{2+} Activated Cl^- Channel Ano6 in Platelet Activation and Apoptosis

Guoxing Liu[a] Guilai Liu[a] Hong Chen[a] Oliver Borst[a,b] Meinrad Gawaz[b] Andrea Vortkamp[c] Rainer Schreiber[d] Karl Kunzelmann[d] Florian Lang[a]

Departments of [a]Physiology and [b]Cardiology & Cardiovascular Medicine, Eberhard-Karls-University, Tübingen, [c]Centre of Medical Biotechnology, University of Essen, Essen, [d]Department of Physiology, University of Regensburg, Regensburg, Germany

Key Words
Cytosolic Ca^{2+} concentration • Phosphatidylserine translocation • P-selectin • Integrin • Cell volume

Abstract
Background/Aims: The ubiquitously expressed Ca^{2+} Activated Cl^- Channel Ano6 participates in the stimulation of cell membrane scrambling. Defective Ano6 underlies the Scott syndrome, an inherited bleeding disorder with impaired scrambling of plasma membrane phospholipids. At least in theory, the bleeding disorder of Scott syndrome may result from impaired platelet function. Activators of platelets include thrombin and collagen related peptide (CRP), which trigger increase of cytosolic Ca^{2+}-activity ($[Ca^{2+}]_i$), production of reactive oxygen species (ROS), degranulation, integrin activation, as well as cell shrinkage and phospholipid scrambling of the cell membrane. The present study thus explored whether Ano6 modifies activation-induced alterations of cytosolic Ca^{2+}-activity ($[Ca^{2+}]_i$), degranulation (P-selectin exposure), integrin activation, phosphatidylserine exposure on the platelet surface and platelet volume. ***Methods:*** Platelets from mice lacking Ano6 (*ano6*$^{-/-}$) were compared to platelets from corresponding wild-type mice (*ano6*$^{+/+}$). $[Ca^{2+}]_i$ was estimated from Fluo-3 fluorescence, ROS from DCFDA fluorescence, degranulation from P-selectin abundance, integrin activation from $\alpha_{IIb}\beta_3$-integrin abundance, phosphatidylserine abundance from annexin-V-binding, and cell volume from forward scatter. ***Results:*** Platelet number in blood was slightly higher in *ano6*$^{-/-}$ mice than in *ano6*$^{+/+}$ mice. Without activation $[Ca^{2+}]_i$ and volume were similar in *ano6*$^{-/-}$ and *ano6*$^{+/+}$ platelets as well as ROS abundance, P-selectin abundance, $\alpha_{IIb}\beta_3$ integrin activation, and phosphatidylserine exposure were negligible in both genotypes. Thrombin (0.01 U/ml) and CRP (2 or 5 µg/ml) increased $[Ca^{2+}]_i$, ROS abundance, platelet degranulation, $\alpha_{IIb}\beta_3$ integrin activation, and triggered annexin-V-binding as well as cell shrinkage, all effects less pronounced in *ano6*$^{-/-}$ than in *ano6*$^{+/+}$ platelets. ***Conclusions:*** Genetic knockout of Ano6 blunts thrombin- and CRP-induced activation and apoptosis of blood platelets.

G. Liu and G. Liu contributed equally and thus share first authorship; K. Kunzelmann and F. Lang contributed equally and thus share last authorship.

Florian Lang

Department of Physiology, University of Tübingen
Gmelinstr. 5, 72076 Tübingen, (Germany)
Tel. +49 7071 29-72194, Fax +49 7071 29-5618, E-Mail florian.lang@uni-tuebingen.de

Introduction

The ubiquitously expressed cell membrane protein Anoctamin 6 (Ano6; TMEM16F gene) may function as outwardly rectifying Ca^{2+}-dependent and volume-regulated Cl^- channel and/or as Ca^{2+}-regulated nonselective Ca^{2+} permeable cation channel [1-5]. Moreover, Ano6 appears to be essential for Ca^{2+}-mediated scrambling of membrane phospholipids and participates in the regulation of cell blebbing and microparticle shedding [1]. Defective Ano6 leads to Scott syndrome, an inherited bleeding disorder with impaired scrambling of plasma membrane phospholipids [1].

Bleeding disorders may result from impaired function of platelets, cells required for primary haemostasis following vascular injury and by the same token instrumental in thrombosis and vascular occlusion [6, 7]. Moreover, platelets contribute to the pathophysiology of vascular inflammation and atherogenesis [6, 8]. Platelets are activated by increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) [9], which may be accomplished by Ca^{2+} release from intracellular stores [10] and subsequent activation of the Ca^{2+} release-activated channel (CRAC) Orai1 (CRACM1) in the plasma membrane [9, 11-13] by the Ca^{2+} sensing stromal interaction molecule 1 (STIM1) [14]. Orai1/STIM1 thus accomplish store-operated calcium entry (SOCE) [15, 16]. Besides platelet activation [9, 10, 12, 17], Ca^{2+} triggers cytoskeletal reorganization [18], cell shrinkage and cell membrane scrambling with translocation of phosphatidylserine to the platelet surface [13]. Agonists stimulating platelets include thrombin and collagen related peptide [19, 20]. The present study explored whether Ano6 participates in the regulation of platelet $[Ca^{2+}]_i$, activation and apoptosis. To this end, the impact of Ano6 deletion on Ca^{2+} entry, platelet activation, cell volume and phospholipid scrambling at the cell membrane was elucidated.

Materials and Methods

Mice

All animal experiments were conducted according to the German law for the welfare of animals and were approved by the authorities of the state of Baden-Württemberg. Experiments were performed with blood platelets isolated from gene targeted mice lacking functional Ano6 (*ano6*$^{-/-}$) and corresponding wild type mice (*ano6*$^{+/+}$). The mice had free access to water and control chow (Ssniff, Soest, Germany). The generation of the mice has been described elsewhere [21].

Preparation of mouse platelets

Platelets were obtained from 10- to 12-week-old mice of either sex. The mice were anesthetized and 800 µl blood was drawn from the retro-orbital plexus into tubes with 200 µl acid-citrate-dextrose buffer before the mice were sacrificed [22]. Blood parameters (see table 1) were analyzed with a KX21-N automatic hematology analyzer (Sysmex). Platelet rich plasma (PRP) was obtained by centrifugation at 260 g for 5 minutes. Afterwards PRP was centrifuged at 640 g for 5 minutes to pellet the platelets. Where necessary apyrase (0.02 U/ml; Sigma-Aldrich) and prostaglandin I_2 (0.5 µM; Calbiochem) were added to the PRP to prevent activation of platelets during isolation. After two washing steps the pellet of washed platelets was resuspended in modified Tyrode-HEPES buffer (pH 7.4, supplemented with 1 mM $CaCl_2$). Where indicated, thrombin (0.01 U/ml, Roche, Basel, Switzerland) or CRP (2 µg/ml or 5 µg/ml, kindly provided by R.Farndale, University of Cambridge, Cambridge, UK) were added [23]. Lower agonist concentrations have been used to activate the platelets, whereas higher agonist concentrations were required to trigger cell membrane scrambling.

Quantification of reactive oxygen species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). Washed platelets were incubated for 15 minutes (37°C) with 0.01 U/ml thrombin and 2 µg/ml CRP, and washed two times with 350 µl Tyrode buffer after stimulation by agonists. They were subsequently stained with DCFDA (10 µM; Sigma, Schnelldorf, Germany) in Tyrode buffer at 37°C for 30 min and washed once in 150 µl Tyrode buffer. The DCFDA-loaded platelets were re-suspended in 200 µl Tyrode buffer and ROS-dependent fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a BD FACS Calibur.

Cytosolic calcium measurements

For the measurement of the intracellular Ca^{2+} concentration the platelet preparation was washed once in Tyrode buffer (pH 7.4), stained with 3 µM Fluo-3AM (Biotinium, USA) in the same buffer and incubated at 37°C for 30 minutes. Following the indicated experimental treatment, fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm utilizing a BD FACS Calibur (BD Biosciences, Heidelberg, Germany) [24].

P-selectin and activated integrin abundance

Fluorophore-labeled antibodies were utilized for the detection of P-selectin expression (Wug.E9-FITC) and the active form of $\alpha_{IIb}\beta_3$ integrin (JON/A-PE). Washed mouse platelets ($1x10^6$) were suspended in modified Tyrode buffer (pH 7.4) containing 1 mM $CaCl_2$ and antibodies (1:10 dilution) and subsequently stimulated with thrombin and CRP for the indicated time periods at room temperature (RT). The reaction was stopped by addition of PBS and the samples were immediately analyzed on a BD FACS Calibur.

Phosphatidylserine exposure and forward scatter

In order to determine phosphatidylserine exposure, the platelet preparation was centrifuged at 660 g for 5 minutes followed by washing once with Tyrode buffer (pH 7.4) with 1 mM $CaCl_2$, staining with 1:20 dilution of Annexin-V-FITC (Mabtag, Germany) in Tyrode buffer (pH 7.4) with 2 mM $CaCl_2$ and incubation at 37°C for 20 minutes. Annexin-V-binding reflecting surface exposure of phosphatidylserine was evaluated by flow cytometry utilizing a BD FACS Calibur. In parallel, the forward scatter (FSC) of the platelets was determined by flow cytometry as a measure of platelet size.

Platelet aggregation

Aggregation was determined utilizing flow cytometry as previously described [2]. To this end platelets were labeled with either CD9-APC or CD9-PE monoclonal antibodies (1:100 dilution, Abcam) for 15 minutes at room temperature. Following incubation, differently labeled samples were washed twice, mixed 1:1 and then pre-incubated at 37°C while shaking at 600 rpm for 10 min. Pre-incubated platelets were activated with thrombin or collagen related peptide at 37°C while shaking at 1000 rpm. At the indicated time points, samples were fixed by addition of 0.5% paraformaldehyde (Carl Roth, Germany) in phosphate-buffered saline. The fixed samples were measured utilizing a BD FACSCalibur (BD Biosciences, Heidelberg, Germany). For quantification, a quadrant was set in the dot plot of respective channels on non-stimulated platelets. The appearance of double-colored events in the upper right quadrant (Q2) was quantified as percentage of total amount of labeled events (Q1+Q2+Q4) at every time point analyzed.

Statistical analysis

Data are provided as means ± SEM; *n* represents the number of independent experiments. All data were tested for significance using ANOVA with Tukey's test as post-test or unpaired student's t-test as appropriate. Results with $p<0.05$ were considered statistically significant.

Results

The present study explored whether Ano6 modifies platelet activation and apoptosis. To this end, murine platelets were isolated from gene targeted mice lacking functional Ano6 (*ano6*$^{-/-}$) and corresponding wild type mice (*ano6*$^{+/+}$). As listed in Table 1, the platelet count was slightly, but significantly ($p<0.05$) higher in *ano6*$^{-/-}$ than in *ano6*$^{+/+}$ mice. Platelet volume, erythrocyte count, erythrocyte volume, hemoglobin concentration and white blood cell count were virtually identical in *ano6*$^{-/-}$ and *ano6*$^{+/+}$ mice (Table 1).

Fluo-3 fluorescence was employed to determine cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). Without treatment $[Ca^{2+}]_i$ was similar in platelets isolated from *ano6*$^{+/+}$ mice and *ano6*$^{-/-}$ mice (Fig. 1A, D). Activation of platelets with thrombin (100 seconds, 0.01 U/ml) was followed by a significant increase of $[Ca^{2+}]_i$ in both, *ano6*$^{+/+}$ and *ano6*$^{-/-}$ platelets. The effect was, however, significantly less pronounced in *ano6*$^{-/-}$ platelets than in *ano6*$^{+/+}$ platelets (Fig. 1B, D). Activation of the platelets with collagen related peptide CRP (100 seconds, 2 µg/ml) was again followed by a significant increase of $[Ca^{2+}]_i$ in

Table 1. Blood count in Ano6 deficient mice *(ano6$^{-/-}$)* and corresponding wild type mice *(ano6$^{+/+}$)*. Arithmetic means ± SEM (n = 6-9), *(p<0.05) indicates statistically significant difference from *ano6$^{+/+}$* mice

	ano6$^{+/+}$	*ano6$^{-/-}$*
Platelet number (10^3/µl)	*1086 ± 53.58*	*1252 ± 39.59**
Mean platelet volume (MPV) (fl)	*6.40 ± 0.11*	*6.27 ± 0.10*
Platelet Distribution Width (PDW) (fl)	*7.33 ± 0.17*	*7.11 ± 0.17*
Platelet larger cell ratio (P-LCR) (%)	*4.68 ± 0.51*	*4.21 ± 0.48*
Erythrocyte number (10^6/µl)	*11.25 ± 2.11*	*10.56 ± 1.35*
Hemoglobin (g/dl)	*13.90 ± 0.45*	*14.34 ± 0.33*
Hematocrit (%)	*44.35 ± 1.12*	*46.33 ± 0.97*
Mean erythrocyte volume (MCV) (fl)	*51.13 ± 0.25*	*50.99 ± 0.41*
Erythrocyte hemoglobin concentration (MCHC) (g/dl)	*31.25 ± 0.33*	*32.07 ± 0.97*
Hemoglobin/erythrocyte (MCH) (pg)	*15.98 ± 0.23*	*15.80 ± 0.26*

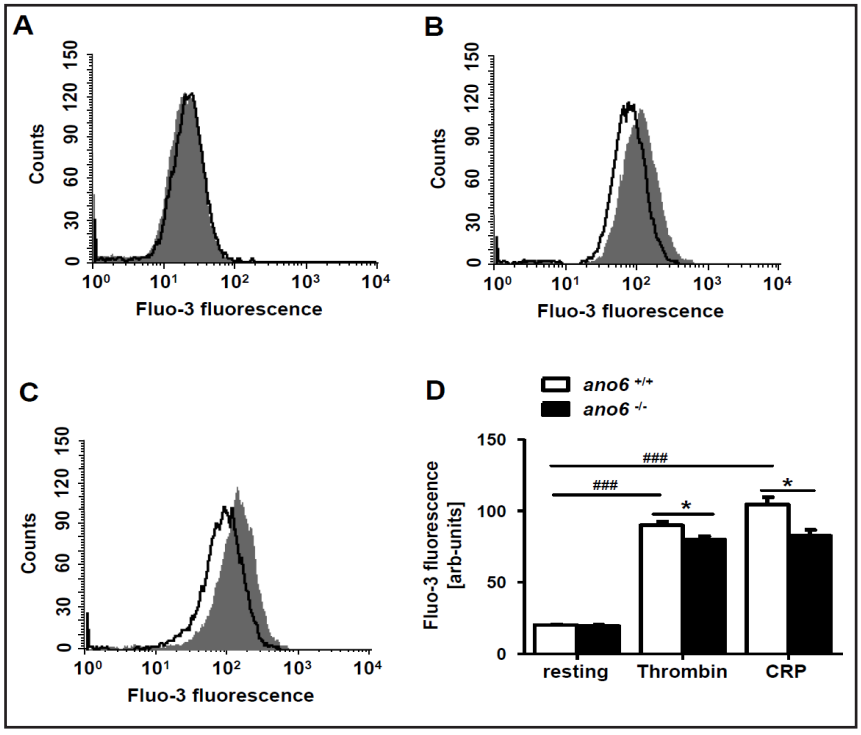

Fig. 1. Ano6 sensitive increase of platelet cytosolic Ca^{2+} concentration. A-C. Original histogram overlays of Fluo-3 fluorescence reflecting cytosolic Ca^{2+} activity in platelets isolated from *ano6$^{+/+}$* mice (grey shadows) and *ano6$^{-/-}$* mice (black lines) without (A) and with 100 sec treatment with (B) thrombin (0.01 U/ml) or (C) collagen related peptide CRP (2 µg/ml). D. Arithmetic means ± SEM (n = 4) of the Fluo-3 fluorescence (arbitrary units) in platelets isolated from *ano6$^{+/+}$* mice (white bars) and *ano6$^{-/-}$* mice (black bars) without treatment (resting), following a 100 sec treatment with thrombin (0.01 U/ml) or collagen related peptide CRP (2 µg/ml). ###(p<0.001) indicates statistically significant difference from absence of thrombin and CRP, *(p<0.05) indicates statistically significant difference from *ano6$^{+/+}$* mice.

both, *ano6$^{+/+}$* and *ano6$^{-/-}$* platelets. The effect was slightly but significantly smaller in *ano6$^{-/-}$* platelets than in *ano6$^{+/+}$* platelets (Fig. 1C, D).

In order to test, whether Ano6 influences oxidative stress, the abundance of reactive oxygen species (ROS) was determined utilizing DCFDA fluorescence. Without treatment ROS was negligible in platelets of both genotypes (Fig. 2A, D). Activation of platelets with thrombin (15 min, 0.01 U/ml) was followed by a significant increase of ROS in both, *ano6$^{+/+}$* and *ano6$^{-/-}$* platelets, The effect was, however, significantly less pronounced in *ano6$^{-/-}$* platelets than in *ano6$^{+/+}$* platelets (Fig. 2B, D). Activation of the platelets with collagen related peptide CRP (15 min, 2 µg/ml) was again followed by a significant increase of ROS in both, *ano6$^{+/+}$* and *ano6$^{-/-}$* platelets. The effect was, however, not significantly different between *ano6$^{-/-}$* platelets and *ano6$^{+/+}$* platelets (Fig. 2C, D).

A next series of experiments addressed the impact of Ano6 on platelet degranulation. The degranulation was apparent from P-selectin abundance at the platelet surface. As illustrated in Fig. 3, P-selectin abundance at the platelet surface was similarly low in untreated platelets

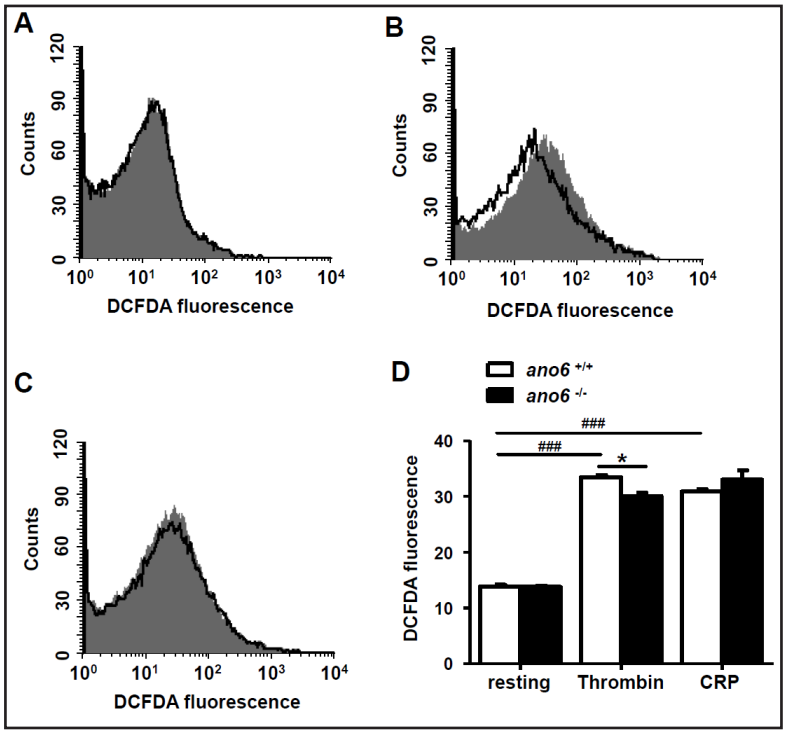

Fig. 2. Ano6 sensitive oxidative stress. A-C. Original histogram overlays of 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence reflecting reactive oxygen species (ROS) in platelets isolated from *ano6*$^{+/+}$ mice (grey shadows) and *ano6*$^{-/-}$ mice (black lines) without (A) and with a 15 min treatment with (B) thrombin (0.01 U/ml) or (C) collagen related peptide CRP (2 µg/ml). D. Arithmetic means ± SEM (n = 3-5) of ROS abundance (arbitrary units) in platelets isolated from *ano6*$^{+/+}$ mice (white bars) and *ano6*$^{-/-}$ mice (black bars) without treatment (resting) and following a 15 min treatment with thrombin (0.01 U/ml) or collagen related peptide CRP (2 µg/ml). ###(p<0.001) indicates statistically significant difference from absence of thrombin and CRP, *(p<0.05) indicates statistically significant difference from *ano6*$^{+/+}$ mice.

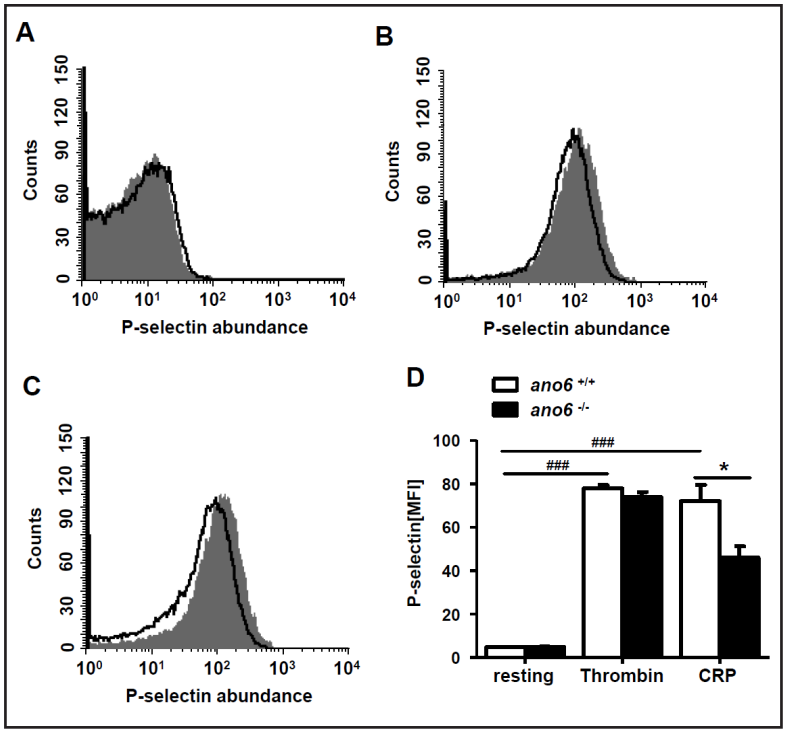

Fig. 3. Ano6 sensitive platelet degranulation. A-C. Original histogram overlays of P-selectin related fluorescence in platelets isolated from *ano6*$^{+/+}$ mice (grey shadows) and *ano6*$^{-/-}$ mice (black lines) without (A) and with a 15 min treatment with (B) thrombin (0.01 U/ml) or (C) collagen related peptide CRP (2 µg/ml). D. Arithmetic means ± SEM (n = 4-7) of the P-selectin related fluorescence (arbitrary units) in platelets isolated from *ano6*$^{+/+}$ mice (white bars) and *ano6*$^{-/-}$ mice (black bars) without treatment (resting) and following a 15 min treatment with thrombin (0.01 U/ml) or collagen related peptide CRP (2 µg/ml). ###(p<0.001) indicates statistically significant difference from absence of thrombin and CRP, *(p<0.05) indicates statistically significant difference from *ano6*$^{+/+}$ mice.

isolated from *ano6*$^{+/+}$ mice and from *ano6*$^{-/-}$ mice. Activation of the platelets with thrombin (15 min, 0.01 U/ml) was followed by a significant increase of P-selectin abundance in both, *ano6*$^{+/+}$ and

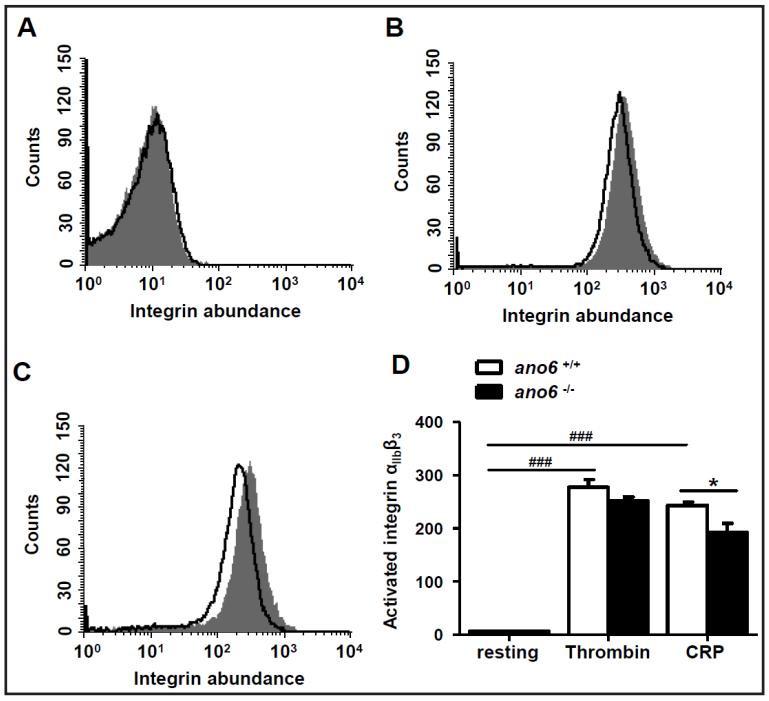

Fig. 4. Ano6 sensitive integrin $\alpha_{IIb}\beta_3$ activation. A-C. Original histogram overlays of the $\alpha_{IIb}\beta_3$ integrin related fluorescence in platelets isolated from *ano6*$^{+/+}$ mice (grey shadows) and *ano6*$^{-/-}$ mice (black lines) without (A) and with a 15 min treatment with (B) thrombin (0.01 U/ml) or (C) collagen related peptide CRP (2 µg/ml). D. Arithmetic means ± SEM (n = 4-6) of the $\alpha_{IIb}\beta_3$ integrin related fluorescence (arbitrary units) in platelets isolated from *ano6*$^{+/+}$ mice (white bars) and *ano6*$^{-/-}$ mice (black bars) without treatment (resting) and following a 15 min treatment with thrombin (0.01 U/ml) or collagen related peptide CRP (2 µg/ml). ###(p<0.001) indicates statistically significant difference from absence of thrombin and CRP, *(p<0.05) indicates statistically significant difference from *ano6*$^{+/+}$ mice.

ano6$^{-/-}$ platelets. The effect tended to be slightly smaller in *ano6*$^{-/-}$ platelets than in *ano6*$^{+/+}$ platelets, a difference, however, not reaching statistical significance (Fig. 3). Activation of the platelets with collagen related peptide CRP (15 min, 2 µg/ml) was again followed by a significant increase of P-selectin abundance in both, *ano6*$^{+/+}$ and *ano6*$^{-/-}$ platelets. The effect was, however, significantly less pronounced in *ano6*$^{-/-}$ platelets than in *ano6*$^{+/+}$ platelets (Fig. 3).

Further experiments addressed the impact of Ano6 on integrin $\alpha_{IIb}\beta_3$ activation. As illustrated in Fig. 4, the abundance of activated integrin $\alpha_{IIb}\beta_3$ at the platelet surface was similarly low in untreated platelets isolated from *ano6*$^{+/+}$ mice and *ano6*$^{-/-}$ mice. Activation of the platelets with thrombin (15 min, 0.01 U/ml) was followed by a significant increase of the abundance of activated integrin $\alpha_{IIb}\beta_3$ in both, *ano6*$^{+/+}$ and *ano6*$^{-/-}$ platelets. The effect tended to be smaller in *ano6*$^{-/-}$ platelets than in *ano6*$^{+/+}$ platelets, a difference, however, not reaching statistical significance (Fig. 4). Activation of the platelets with collagen related peptide CRP (15 min, 2 µg/ml) was again followed by a significant increase of integrin $\alpha_{IIb}\beta_3$ activation in both, *ano6*$^{+/+}$ and *ano6*$^{-/-}$ platelets. The effect was significantly less pronounced in *ano6*$^{-/-}$ platelets than in *ano6*$^{+/+}$ platelets (Fig. 4).

A further series of experiments explored the impact of Ano6 on platelet apoptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the platelet surface. Phosphatidylserine abundance was determined utilizing annexin-V-binding and platelet volume utilizing forward scatter. As illustrated in Fig. 5, annexin-V-binding was negligible in untreated platelets isolated from either genotype. Treatment with thrombin (0.01 U/ml) and CRP (5 µg/ml) was followed by an increase of annexin-V-binding in both, *ano6*$^{+/+}$ and *ano6*$^{-/-}$ platelets. The effect was slightly, but significantly, blunted in *ano6*$^{-/-}$ platelets when compared to *ano6*$^{+/+}$ platelets. As shown in Fig. 6, forward scatter was similar in untreated *ano6*$^{-/-}$ platelets and in untreated *ano6*$^{+/+}$ platelets. Treatment with thrombin (15 min, 0.01 U/ml) was followed by a significant decrease of forward scatter in both, *ano6*$^{+/+}$ and *ano6*$^{-/-}$ platelets. The effect was significantly less pronounced in *ano6*$^{-/-}$ platelets than in *ano6*$^{+/+}$ platelets. Treatment with CRP (15 min, 5 µg/ml) was again followed by a significant decrease of forward scatter in both, *ano6*$^{+/+}$ and *ano6*$^{-/-}$ platelets. The effect was again significantly less pronounced in *ano6*$^{-/-}$ platelets than in *ano6*$^{+/+}$ platelets.

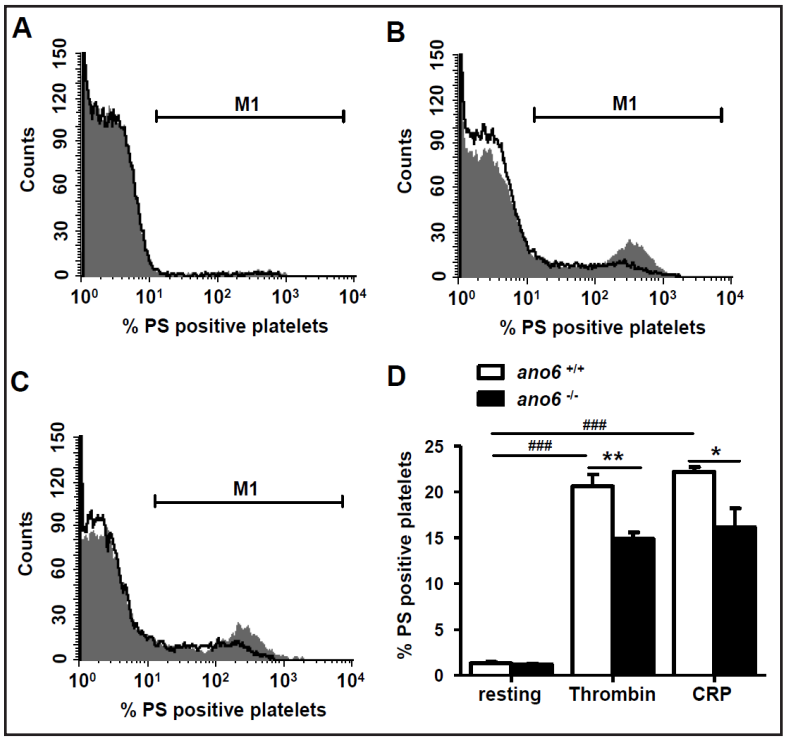

Fig. 5. Ano6 sensitive phosphatidylserine exposure at the platelet surface. A-C. Original histogram overlays of annexin-V binding in platelets isolated from *ano6*$^{+/+}$ mice (grey shadows) and *ano6*$^{-/-}$ mice (black lines) without (A) and with a 15 min treatment with (B) thrombin (0.01 U/ml) or (C) collagen related peptide CRP (5 µg/ml). D. Arithmetic means ± SEM (n = 4-6) of annexin-V binding (arbitrary units) in platelets isolated from *ano6*$^{+/+}$ mice (white bars) and *ano6*$^{-/-}$ mice (black bars) without treatment (resting) and following a 15 min treatment with thrombin (0.01 U/ml) or collagen related peptide CRP (5 µg/ml). ###(p<0.001) indicates statistically significant difference from absence of thrombin and CRP, *(p<0.05), ** (p<0.01) indicates statistically significant difference from *ano6*$^{+/+}$ mice.

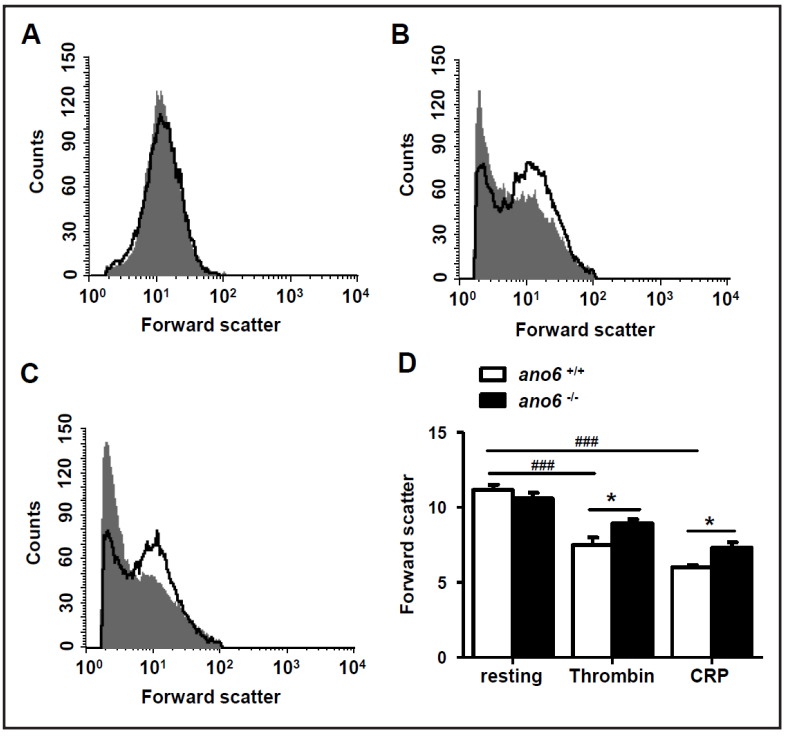

Fig. 6. Ano6 sensitive platelet forward scatter. A-C. Original histogram overlays of forward scatter in platelets isolated from *ano6*$^{+/+}$ mice (grey shadows) and *ano6*$^{-/-}$ mice (black lines) without (A) and with a 15 min treatment with (B) thrombin (0.01 U/ml) or (C) collagen related peptide CRP (5 µg/ml). D. Arithmetic means ± SEM (n = 4-6) of the forward scatter of platelets isolated from *ano6*$^{+/+}$ mice (white bars) and *ano6*$^{-/-}$ mice (black bars) without treatment (resting) and following a 15 min treatment with thrombin (0.01 U/ml) or collagen related peptide CRP (5 µg/ml). ###(p<0.001) indicates statistically significant difference from absence of thrombin and CRP, *(p<0.05) indicates statistically significant difference from *ano6*$^{+/+}$ mice.

To elucidate the effect of Ano6 on platelet aggregation, platelets were labeled with two distinct dyes and the coincidence of the two dyes estimated by flow cytometry. As illustrated in Fig. 7, aggregation of resting platelets was similarly low in *ano6*$^{+/+}$ and *ano6*$^{-/-}$ platelets, and

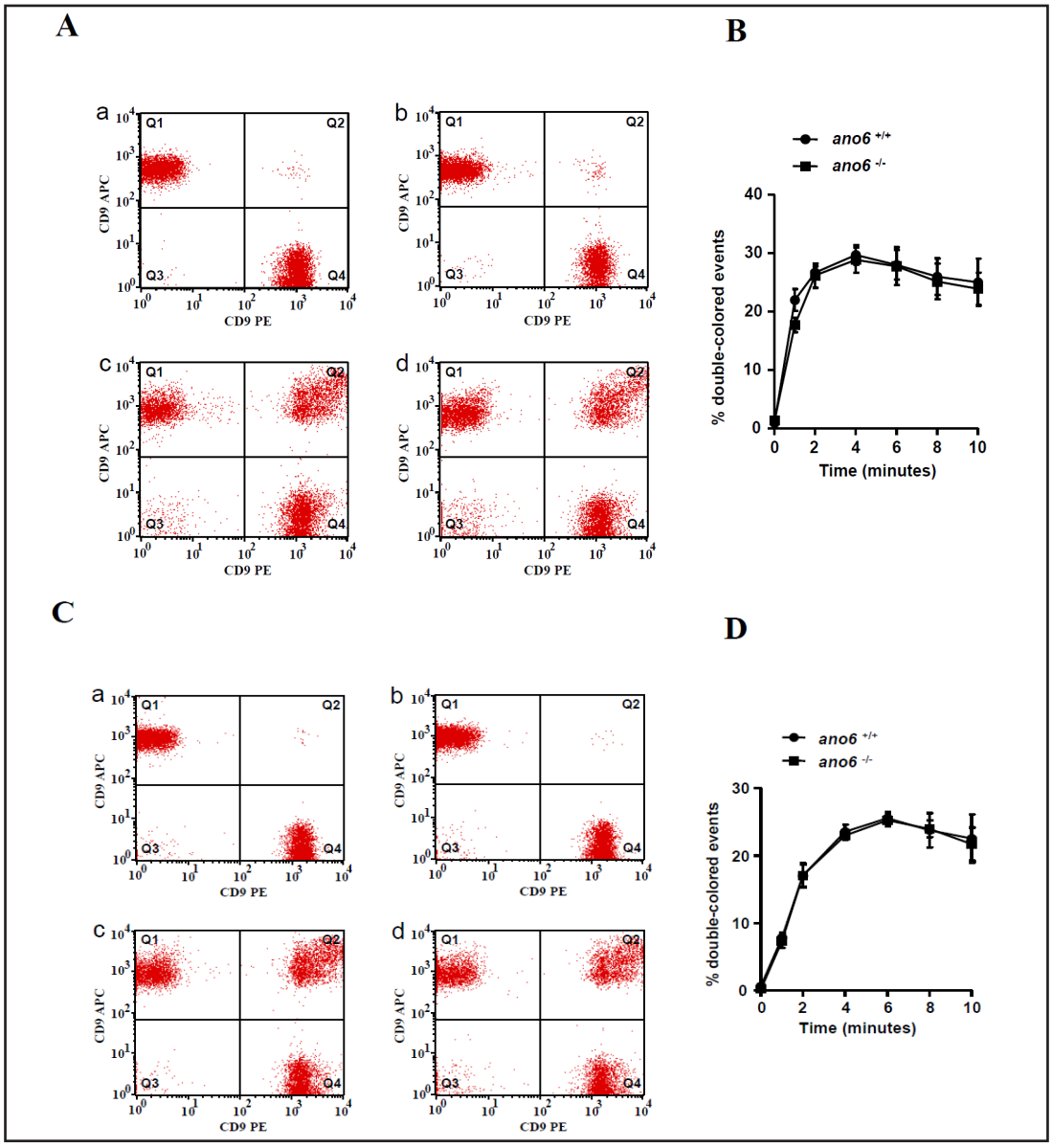

Fig. 7. Ano6 insensitive platelet aggregation. A. Original dot blots reflecting platelet aggregation in platelets isolated from *ano6*$^{+/+}$ mice (a, c) and *ano6*$^{-/-}$ mice (b, d), and subsequent treatment with 0.005 U/ml thrombin in 0 min (a, b) and 10 min (c, d). B. Arithmetic means ± SEM (n = 4) of platelet aggregation in platelets isolated from *ano6*$^{+/+}$ mice (black circles) and *ano6*$^{-/-}$ mice (black square) as a function of time after addition of thrombin (0.005 U/ml). C. Original dot blots reflecting platelet aggregation in platelets isolated from *ano6*$^{+/+}$ mice (a, c) and *ano6*$^{-/-}$ mice (b, d), and subsequent treatment with collagen related peptide CRP (2 µg/ml) in 0 min (a, b) and 10 min (c, d). D. Arithmetic means ± SEM (n = 4) of platelet aggregation in platelets isolated from *ano6*$^{+/+}$ mice (black circles) and *ano6*$^{-/-}$ mice (black square) as a function of time after addition of CRP (2 µg/ml).

significantly increased in a few minutes to similarly high levels in *ano6*$^{+/+}$ and *ano6*$^{-/-}$ platelets following treatment with 0.005 U/ml thrombin or 2 µg/ml CRP.

Discussion

The present observations disclose a subtle but significant impact of Ano6 on platelet activation and apoptosis following thrombin or collagen related peptide (CRP) treatment.

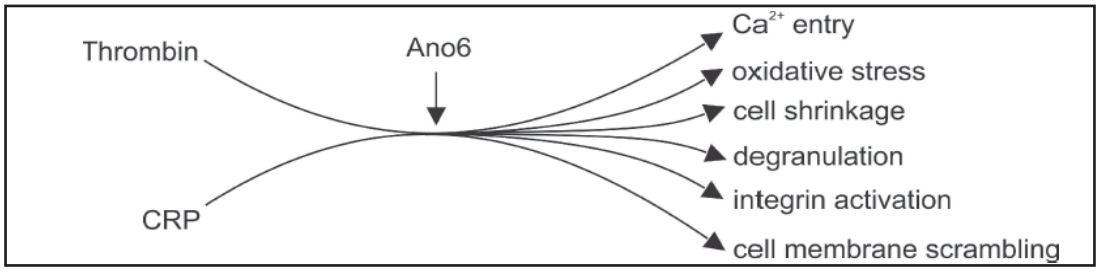

Fig. 8. Synopsis of Ano6 sensitive platelet functions.

Prior to activation, cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), reactive oxygen species (ROS), P-selectin abundance, $\alpha_{IIb}\beta_3$ integrin activation, phosphatidylserine exposure and cell volume were similar in platelets isolated from gene targeted mice lacking Ano6 (*ano6*$^{-/-}$) and in platelets from corresponding wild-type mice (*ano6*$^{+/+}$). Thrombin and CRP increased $[Ca^{2+}]_i$, as well as ROS abundance, and triggered platelet degranulation as well as $\alpha_{IIb}\beta_3$ integrin activation, effects less pronounced in *ano6*$^{-/-}$ than in *ano6*$^{+/+}$ platelets. A key event in the activation of platelets is increase of $[Ca^{2+}]_i$ [13], which triggers platelet degranulation, adhesion and aggregation thus supporting development of thrombosis [9].

Thrombin and CRP further trigger phosphatidylserine translocation and cell shrinkage, key events in apoptosis limiting the life span of circulating platelets [17]. The stimulation of platelet apoptosis may be similarly secondary to increase of $[Ca^{2+}]_i$ [13], which is known to stimulate cell membrane phospholipid scrambling with phosphatidylserine translocation [8, 25-27]. According to the present observations, the phosphatidylserine exposure depends in part on the presence of Ano6. The thrombin and CRP induced cell shrinkage again depends in part on the presence of Ano6. It is conceivable that the Cl^- channel function of Ano6 [1] mediates Cl^- exit and thus supports the loss of ions and osmotically obliged water [28].

A blunted Ca^{2+} increase was observed in *ano6*$^{-/-}$ platelets upon stimulation with thrombin and CRP. It is important to note that Ano6 has been described as a nonselective and Ca^{2+} permeable channel, which could be relevant for the present observations [29-31]. It should be pointed out, however that the scrambling defect in *ano6*$^{-/-}$ platelets is not due to defective Ca^{2+} influx through Ano6 [1]. It is possible that Ano6 affects intracellular Ca^{2+} signaling indirectly, as shown earlier for Ano1 [32].

Phosphatidylserine exposing platelets bind to and are engulfed by macrophages [33]. Phosphatidylserine at the platelet surface further stimulates coagulation and thus contributes to the triggering of hemostasis [34]. Phosphatidylserine exposure further stimulates thrombin formation and platelet pro-coagulant activity [25-27, 35].

The platelet number in blood was higher in *ano6*$^{-/-}$ mice than in *ano6*$^{+/+}$mice. It is tempting to speculate that the decreased sensitivity of phosphatidylserine translocation to thrombin and CRP is followed by decreased clearance of phosphatidylserine exposing platelets from circulating blood.

In conclusion, Ano6 contributes to both, platelet activation and platelet apoptosis (Fig. 8). Ano6 deficiency blunts the stimulating effect of thrombin and CRP on Ca^{2+} entry, platelet degranulation and integrin activation, phosphatidylserine translocation and platelet shrinkage. Thus, Ano6 is a novel element in the regulation of platelet function and survival.

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Disclosure Statement

The authors of this manuscript state that they have no conflicts of interest to declare.

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